Volume 232, number 2, 409–413 FEB 05884 May 1988

Microinjection of catalytic subunit of cyclic AMP-dependent protein kinases triggers acute morphological changes in thyroid epithelial cells

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Received 23 March 1988

In dog thyroid epithelial cells in primary culture, thyrotropin acting through cyclic AMP induced rapid morphological changes associated with complete disruption of actin containing stress fibers. This modification preceded cell retraction and rounding up. These morphological effects were also induced by glass capillary microinjection of purified catalytic subunit of cAMP-dependent protein kinase. This provides the first direct evidence in intact cells that catalytic subunit, which is released upon activation of cAMP-dependent protein kinases, is responsible for cAMP-dependent morphological transformation.

cyclic AMP-dependent protein kinase; Microinjection; Actin; Stress fiber; Thyroid cell

1. INTRODUCTION

Cyclic AMP (cAMP)-dependent protein kinases (PKA) are the major transducers for agents regulating intracellular levels of cAMP. Kinase holoenzymes are tetrameric, with two catalytic subunits (C) and a regulatory subunit dimer (R) [1]. Upon activation, freed C subunits mediate the cAMP effects by phosphorylating specific proteins [2]. However, definitive demonstration of this mechanism has generally not been provided in intact cells, especially on processes affecting morphology, growth and differentiation. Recently, evidence for alternative mechanisms has appeared, suggesting additional roles for R subunits such as inhibition of protein phosphatases [3–5] and stimulation of transcription [6,7], or direct action of cyclic nucleotides on ion channels [8].

In several epithelial and nonepithelial cells in culture, one of the most dramatic effects of agents increasing cellular cAMP is a rapid disorganization of the actin microfilament network (stress fibers) followed by cytoplasmic retraction and rounding [9–15]. This might affect various processes including membrane movements, signal transduction, cell communication, intracellular transport, and cell function. This effect is especially striking in primary cultures of dog thyroid epithelial cells in response to thyrotropin (TSH) [16–18]. We directly addressed the question of whether acute morphological and cytoskeletal changes induced by cAMP are dependent on C subunit activity by microinjecting this purified subunit in thyroid cells.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

2.1. *Cell culture*

Primary cultures of dog thyroid epithelial cells were established as described [19]. Thyroid follicles released by collagenase digestion of the minced tissue were seeded and cultured in plastic Petri dishes in a serum-free medium containing insulin (5 μg/ml). Cells were used 4 days after seeding, when follicles had collapsed, developing colonies of epithelial cells in monolayer.

2.2. *Microinjection of purified catalytic subunit of PKA*

The catalytic subunit of bovine heart PKA was purified as described [20]. The enzyme was stable for several months and was homogeneous by SDS gel electrophoresis. It did not contain measurable proteolytic or protein phosphatase activity. The pure enzyme was dialysed extensively against the following buffer: 5 mM Hepes, pH 7.2, 110 mM KCl, 0.05 mM DTT. Protein concentration was 11 mg/ml. Injection of PKA subunit was performed with glass capillaries according to the method described originally by Graesmann [21]. Cells to be injected were grown in 6 cm Petri dishes. For a typical experiment 100 cells were injected in a field delimited on the bottom of the dish. The volume injected into each cell was 5×10^{-5} nl average value.

2.3. *Culture morphology and indirect immunofluorescence staining*

The same microscope field was observed by phase contrast microscopy, before treatment of cells and at different times after microinjection or TSH addition. 20 or 210 min after microinjection or TSH addition, cells were fixed in methanol for 7 min at -20°C. After washing in TBS (Tris 10 mM, NaCl 155 mM, pH 8.2), Triton X-100 (0.15% in TBS) was added for 10 min at room temperature. Subsequent rinses were followed by addition of normal sheep serum (1/20 in TBS containing 0.1% bovine serum albumin (BSA)) for 30 min at room temperature. The serum was drained off and replaced by mouse monoclonal antiactin (1/250 in TBS with 0.1% BSA – N 350 from Amersham) overnight at room temperature. After incubation, the cells were washed 3 times and reacted with fluorescein conjugated-sheep anti-mouse immunoglobulin G (1/200 in TBS with 0.1% BSA – Amersham). After further thorough washings, the Petri dishes were mounted in Gelvatol (polyvinyl alcohol) (Monsanto) containing 100 mg/ml DABCO (1,4-diazobicyclo(2,2,2)octane 97% – Janssen) in order to delay the fluorescence fading. Cells were viewed on a Zeiss epifluorescence microscope and were photographed with 400 iso Fuji films.

3. RESULTS

Four-day-old thyroid cells were organized in monolayer made up of colonies of cells remaining in close contact. They displayed a dense filamentous actin network: actin decorated stress fibers spanned throughout the entire cell length. They inserted into sites near the plasma membrane (fig.1A). The addition of TSH (1 mU/ml) to thyroid epithelial cells in monolayer triggered acute morphological changes, as observed by phase contrast microscopy (not shown). Within 20 min, membrane ruffles and scalopping of the cell periphery were evident. Progressive cytoplasmic retraction was then seen, resulting in complete arborization of the cells which remained associated by dendrite-like cytoplasmic extensions, 3–12 h after TSH addition.

Indirect immunofluorescence studies using antiactin monoclonal antibody showed that early morphological changes were associated with disorganization of prominent actin containing stress fibers (fig.1B). As son as 20 min after TSH addition, actin was visualized as finely granulated cytoplasmic dots as well as a stronger staining along cell periphery. This redistribution clearly preceded cytoplasmic retraction (fig.1C) and was therefore assumed to be causative for this dramatic morphological response to TSH. Identical effects were provoked by forskolin (10^{-5} M), a nonspecific adenylate cyclase activator, and by dibutyryl cAMP (10^{-4} M) (not shown), indicating that the effects of TSH are mediated by cAMP elevation.

In order to directly test the possibility that the effects of TSH and cAMP on cytoskeleton and morphology are mediated by the release of active C subunit of PKA, we introduced a solution (11 mg/ml) of purified bovine heart C subunit in thyroid cell cytoplasms by glass capillary microinjection. As shown in fig.2, like TSH addition, microinjection of C subunit induced acute morphological changes with membrane ruffles (fig.2B), associated with disorganization of stress fibers, and staining of actin at cell periphery (fig.1D). The effect was prolonged by complete retraction and rounding up of microinjected cells (figs.1E,2D). The morphological response subsisted for several hours, but 24 h after microinjection of C subunit, cells had regained the morphology of untreated cells. Nonmicroinjected cells and cells microinjected with buffer did not present these morphological and cytoskeletal responses (not shown). About 25% of cells were killed by the traumatism due to microinjection, but they were clearly distinguished from cells undergoing morphological responses (fig.2D).

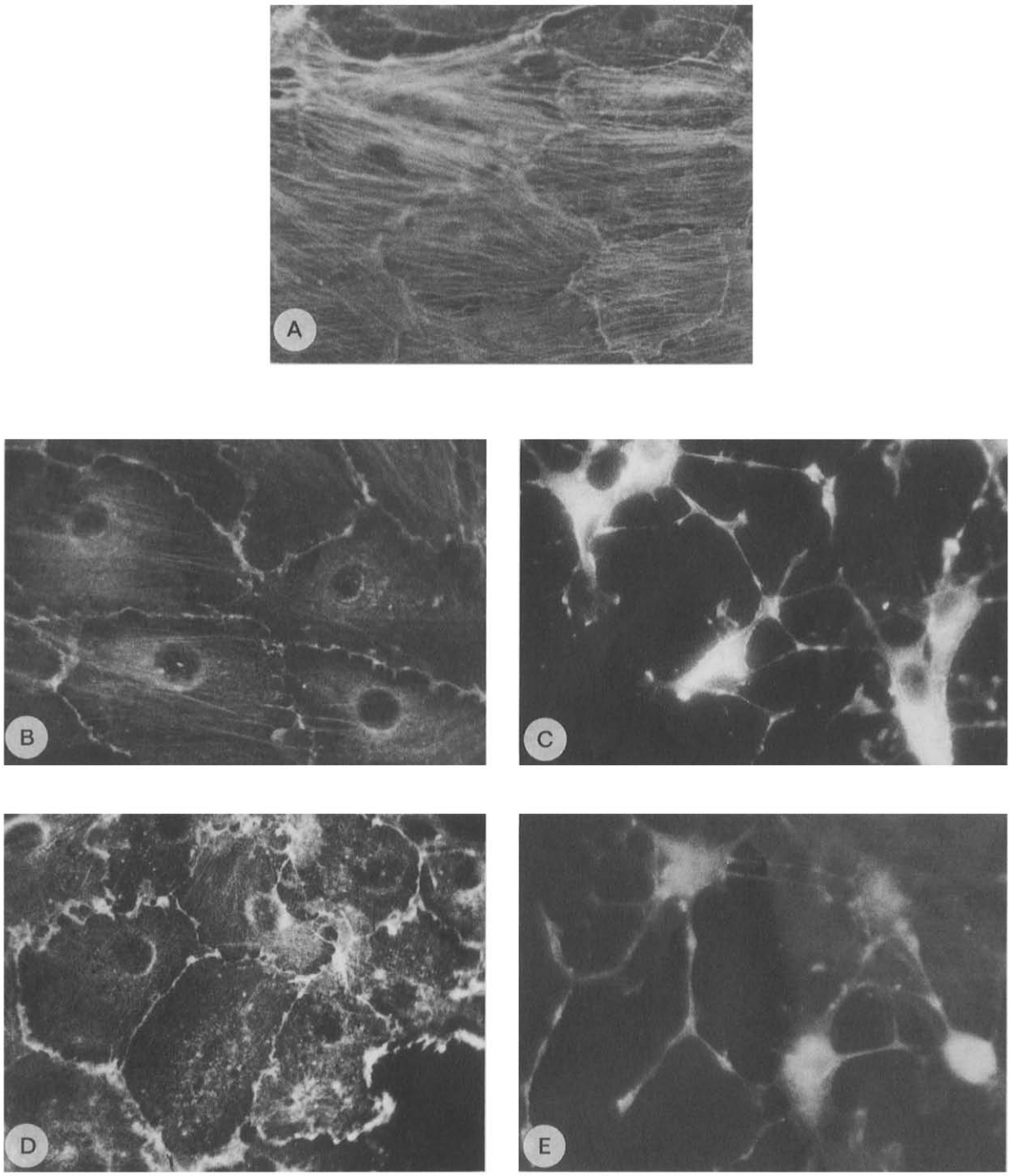

Fig.1. Indirect immunofluorescence of thyroid cells stained with antibody to actin. Cells were incubated in control medium (A), and with TSH (1 mU/ml) for 20 min (B) or 3.5 h (C). Cells were also fixed for 20 min (D) and 3.5 h (E) after microinjection of the C subunit of PKA. × 500.

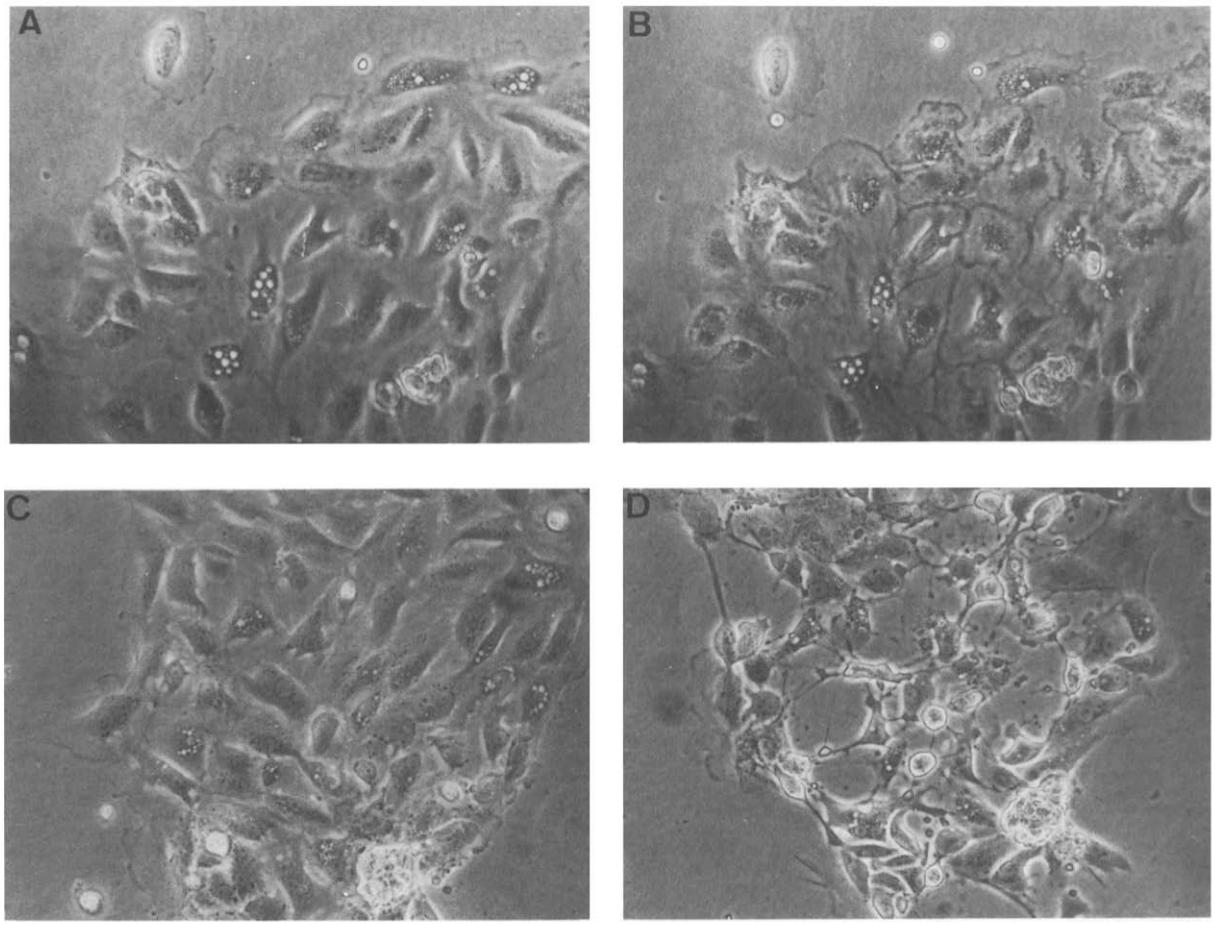

Fig.2. Phase contrast microscopy of living thyroid cells. Cells were photographed before (A,C) and 20 min (B) or 3.5 h (D) after microinjection of the C subunit of PKA. A, B and C, D show the same cells before and after microinjection. × 210.

4. DISCUSSION

Profound changes in morphology caused by the rapid disruption of actin containing stress fibers have a relatively wide occurrence in different cell types in culture submitted to hormones acting through cAMP elevation [9–18]. They share many similarities with morphological transformation caused by tumorigenic retrovirus infections [20] or induced by tumor promoters [23]. Their real significance regarding the function of normal cells remains merely conjectural. Major cellular processes are, however, assumed to be dependent on cytoskeleton integrity, cytoskeleton-membrane associations and intercellular contacts.

At present very little is known about mechanisms involved in the hormone-induced, cAMP-dependent morphological transformation. As it is very rapid and not inhibited by protein synthesis inhibitors (unpublished results), it is not dependent on modifications of gene expression. In order to directly assess the involvement of PKA in the acute effects of TSH and cAMP on actin network organization and cell morphology, we microinjected purified C subunit of PKA in thyroid cells. This was done with the assumption that increasing cytoplasmic content of C subunit to about 10^{-5} M would saturate the inhibitory capacity of endogenous R subunits, and thereby mimic the activation of PKA by cAMP.

We thus observed that free C subunit had the capacity to induce the same effects as triggered by

TSH on microfilament organization and cell morphology. This demonstrates the feasibility of this approach to test the role of the C subunit of PKA in intact cells.

Proteins whose phosphorylation by C subunit of PKA might cause such dramatic reorganization of cytoskeleton are not known. They might be actin-associated proteins, since cytoskeletal changes induced by protein kinase C activators or infection with tumorigenesis retroviruses are correlated with increased phosphorylation of vinculin [24], talin [25], calpactin [26]. These proteins are involved in stabilization and membrane anchorage of microfilaments. Alternatively, C subunit phosphorylates the myosin light chain kinase [27,28], which could lead to the decrease in myosin light chain phosphorylation that is observed together with stress fiber disruption in dog thyroid cells [29,30].

Acknowledgements: The authors are grateful to Dr P. Neve for advice and to Mrs D. Leemans for preparation of the manuscript. This work was made possible thanks to a grant of the Ministère de la Politique Scientifique (Action Concertée). M.A. was supported by a grant of the Belgian FRSM.

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